Comm.
Dr. Gardner
Dr. Jacobson
Dr. Meier
Dr. Sommers

CHRONIC PULMONARY DISEASES
RENEWAL PROPOSAL

#573BR2
#573BR1-10/1/72 - 9/31/73
#573B -10/1/71 - 9/31/72
Contract # 7 - 10/1/70 - 9/31/71
573A-10/1/69 - 9/31/70
573-9/1/66 - 9/31/69

The Effects of Fresh Cigarette Smoke Inhalation on the Respiratory Tract of [illegible]

COUNCIL FOR TOBACCO RESEARCH - U.S.A.
110 East 59th Street, New York, N.Y.
10022

BY

UNIVERSITY OF SOUTHERN CALIFORNIA
University Park
Los Angeles, California 90007

Principal Investigator:

Clayton G. Loosli, M.D., Ph.D.
Hastings Professor of
Pathology

Approved for the
Department of Pathology:

Nancy E. Warner, M.D.
Chairman, Department of
Pathology

Approved for the University by:

Zohrab A. Kaprielian,
Vice President, Academic
Administration and Research

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 East 59th Street
New York, N.Y. 10022
(212) 421-6534

Application for Renewal of Research Grant
(Use extra pages as needed)

First Renewal Second Renewal Date: Jan. 24, 1973

1. Principal Investigator (give title and degrees):
   Clayton G. Loosli, M.D., Ph.D. Hastings Professor of Medicine and Pathology
   Helgard Niewisch, D.V.M., Research Associate in Pathology
   Kuen-Shan Hung, Ph.D., Assistant Professor of Anatomy and Pathology.

2. Institution and address:
   The University of Southern California
   University Park
   Los Angeles, California 90007

3. Department(s) where research will be done or collaboration provided:
   USC School of Medicine
   Department of Pathology
   2025 Zonal Avenue
   Los Angeles, California 90033

4. Short title of study:
   Effects of Fresh Cigarette Smoke Inhalation on the Respiratory Tract of Mice

5. Proposed renewal date:
   July 1, 1973

6. How results to date have changed earlier specific research aims:
   Basically, there are no changes in the objectives set forth in the application two years ago. However, in order to concentrate solely on the effects of inhalation of fresh tobacco smoke alone, the "synthetic smog" exposure studies will be discontinued as of June 30th. The SS exposure room will be used as an additional filtered air room where the additional smoke exposed and control mice will be housed.

7. How results to date have changed earlier working hypothesis:
   There is no basic change in working hypothesis. Four strains of mice are being exposed and sacrificed periodically and the lungs examined histologically, histochemically, and by E.M. procedures. Current findings strongly suggest that a C type virus particle is responsible for bronchial epithelial metaplastic growth. What activated the virus is not known. Studies will be directed toward identification of the viral agent or

or agents, and transmission of virus in SPF mice under strict conditions of isolation. Also macrophage accumulation and retention in the lungs of smoke exposed animals suggests that they may contribute to lung tissue breakdown. Studies will be directed toward ellucidating the role of pulmonary macrophages in the development of emphysematous-like lung lesions.

8. Any additional facilities now required? Describe briefly:
No additional facilities or equipment except a CO_2 incubator is required. As is the case now, the Air Pollution Building will continue to be used for exposing and housing both smoke exposed and control mice. Synthetic smog (SS) studies will be discontinued. The SS room supplied with filtered air will be used to house the sham and non-smoking control animals. For viral transmission studies, the germfree isolator units in the germfree facility will be employed to house the mice inoculated with suspected infectious material. Tissue culture facilities are available for culture of lungs and other organs of mice suspected of harboring virus. Serological procedures for identifying antibodies to suspected viruses will be employed in identifying the virus.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:
Dr. Bernard Hanes, not listed on the grant, will continue to serve as a consultant. Dr. Hung continues to spend essentially all his time on electron microscopic examination of lung tissue. Dr. Helgard Niewisch, D.V.M., who joined our group last September, will continue to supervise the smoke exposure program and the smoking machine operators - Paul LeVangie, Peter Bondante, and Mrs. Alicia Navarro. The smoking machine operators are also being trained in various laboratory procedures, to clean, wrap and sterilize bacteriological and virological equipment and to prepare the food for the germfree mice. They will make valuable assistants when the studies on the nature and identification of the virus begin.

Dr. Niewisch, having been in the Department of Microbiology for 6 years, has competence in bacteriological and virological procedures. She will participate in the studies associated with isolation and identification of the virus seen in the E.M. photographs, in association with the epithelial metaplastic growth. Drs. Richard O'Brien and John Parker have an active tissue culture laboratory and have offered to assist us and train, if necessary, the virologist, which at this point in time, has to be recruited.

John Hardy, M.S. (Exp. Path.), Sherman Stinson, M.S. (Exp. Path.), and Jeanne Joyce, B.S., Germfree laboratory supervisor (not listed on grant), will have responsibility of the animal transmission studies and autopsies of all animals. Mr. Stinson, a trained biochemist, will carry out the phospholipid and surfactant analysis on the lungs.

While the salaries of the technical staff may seem somewhat high, Misses Hertweck, Stone and Serebrin, and the animal caretakers have been with our group for several years. Their salaries are comparative with others in the University with similar skills and experience.

9A. Justification of budget:

For the past 5 years we have had partial support from the E.P.A. for study the effects of "synthetic smog" on the respiratory tract of mice. This grant will terminate March 31, 1973. Also for the past 5 years, the N.I.H. (Laboratory Facilities Division) has underwritten the Germfree Facility. Support for the Germfree laboratory will terminate May 31st. Continued support for our experienced personnel, who have been partially supported by the above government organizations in the past, is absolutely essential for the performance of the studies of the effect of smoke inhalation on the respiratory tract of mice.

No other grant support or applications are pending. Through the Hastings Foundation and the Hughes Employees Give Once Club, the University is contributing significantly in the support of these studies. It will be a great advantage to this study not to have it superimposed on the synthetic smog study. It will aid significantly in planning to have a commitment for an additional two years at the level requested plus 5 percent for living cost increases.

10. Append outline of experimental protocol for the ensuing year:

Since June, 1972, the CTR-Walton Horizontal Smoke Exposure Machines have been in use. At the present time, four are in operation. Four different strains of mice have been employed: CD-1 white, pathogen free (SPF), Ajax white non-pathgen free, C-57 Black non-pathogen free, and Snell non-pathogen free mice. A total of 500 mice are being exposed to fresh cigarette smoke daily. Half will be sacrificed in February and the other half in May, 1973. Synthetic smog studies will be discontinued as of May 31st, 1973.

OUTLINE OF STUDIES FOR 1973-74

A. Continuation of exposure of mice to fresh cigarette smoke from 1A1 low nicotine reference cigarettes. We hope to continue to use the above strains of SPF mice which have been

caesarian derived and barrier maintained to avoid the problem of respiratory infections among the mice. Based on our experience the past year, the maximum daily exposure, which will allow long term survival, will be exposure to fresh smoke from three simultaneously "fired" 1A1 cigarettes once a day. The exposure time after a two-second puff will continue to be 21 seconds, with a purge time of 39 seconds. The Ajax and the CD-1 strains appeared to be somewhat less tolerant and are receiving smoke from two cigarettes a day for 5 days a week. The smoke exposed animals on a once a day regimen maintain their weight. As our interest is primarily in lung changes due to smoke inhalation, we hope to continue to use the low nicotine cigarettes to maintain the maximum exposure up to the limit of tolerance.

The two large filtered air rooms in the air pollution building will be employed to house the mice. One will house the animals subjected to smoke and the other will house the sham and non-sham controls. Sufficient animals from each mouse group will be used so that 18 from each group will be sacrificed at intervals of 3 months. As mice are sacrificed, additional mice of the same age will be added to the smoking regimen. It is our impression so far, that increasingly older mice become less tolerant of fresh cigarette smoke inhalation.

Eighteen animals from the smoke exposed and control groups are chosen to be sacrificed, for it represents the maximum number which can be subjected to smoke inhalation at one time. Several procedures for examining the lungs and other organs will be carried out, as are now being done. Eight mice will be sacrificed for light and electron microscopic study, three for histochemistry, and seven for phospholipid and surfactant analysis. Before sacrifice, all mice will be bled out and the sera stored for immunological studies. Portions of the lung for phospholipid and histochemical analysis will be quickly frozen for subsequent virological analysis. Animals becoming ill during the course of the study will be bled and sacrificed in order to obtain fresh tissue for virological studies.

B. Study of the pulmonary macrophage reaction in Snell mice. Studies thus far indicate that mice inhaling whole fresh smoke from 1A1 reference cigarettes accumulate large foamy macrophages in the pulmonary alveoli in the region of the terminal bronchioles and that these macrophage collections are still present at least one month after smoke exposure is discontinued. It is thought by some that pulmonary macrophages may secrete enzymes which cause lung tissue breakdown, leading to emphysema.

It is assumed that the macrophage reaction in the whole smoke exposed mice is due to the particulate component of smoke. To test this, the following experiment will be carried out: Three groups of Snell mice will be employed. One group will be exposed to fresh whole smoke; one to the gas phase of smoke only, after passing through a Cambridge filter; the third group will serve as controls.

The smoke exposed group will be subjected to a heavy smoking regimen, so that the macrophage reaction can be expected to be present after three months of smoking. Smaller numbers from the three groups will be sacrificed and their lungs examined for the presence of macrophage collections. Others will continue to be exposed to whole and gas phase smoke for another 3 months. At the same time, some animals exposed to whole smoke will be set aside and sacrificed at later periods to note the continued presence of the macrophages in the lungs.

C. Identify the role of viral agent or agents associated with bronchial epithelial metaplasia: During the course of regular smoking schedule of the four strains of mice the latter part of October, 1972, animals from all groups became ill and some died. A sacrifice after 5 and 5 1/2 months revealed widespread pulmonary lesions in all groups - smokers and non-smokers living in the SS and filtered air rooms. The reconstruction of the pathogenesis of the lesions suggests a virus infection of some kind, although no known infected animals or viral agents had ever been introduced into the chambers. The microscopic appearances of the lung lesions were similar in the four strains of mice whether they had been exposed to cigarette smoke or not.

A detailed study of the lung lesions in the Snell mice has been made.

The early lesions show marked destruction of the bronchial epithelium, quite similar to that seen in influenza virus infections. On recovery, the regenerating epithelial membranes become markedly thickened and metaplastic and grow peripherally into the alveolar ducts and associated alveoli as squamous cell masses. The inflammatory cells in the early lesions are polymorphonuclear leukocytes. These become replaced with mononuclear cells in the older lesions. The epithelial lesions are focal in character, involving only a few bronchi with the remaining lung having a normal appearance. Older lesions show degeneration of the squamous cell masses, leaving the alveoli lined with low cuboidal epithelial cells, which stain specifically with G1-6-PDH.

Examination of the lung lesions from one smoke exposed mouse and one unexposed mouse from the Snell group revealed C type viral particles budding from the membranous surfaces of the cells.

It seems certain to us that this C type virus particle is basically involved as the inciting agent in the production of the epithelial growths in the lungs. As all the different groups of animals became ill at the same time whether they were smoke exposed or not, suggests that the virus was disseminated from one group to another as an airborne infection. Undoubtedly the virus is probably still active in the mouse colonies.

The following studies will be attempted:

a.) Rescue of virus through animal to animal transmission. Quickly frozen lung tissue from animals ill with the infection will be ground up and the supernate (hopefully) containing the active virus will be given intranasally to SPF Snell and CD-1 SPF mice which will be kept in Germfree isolators to prevent cross infection.

b.) There will be an attempt to grow the virus in cultures of mouse lung tissue from young SPF Snell and CD-1 mice. Cultures will be examined with the aid of the electron microscope. Drs. O'Brien and Parker have offered to assist in the cell culture preparation of virus activity and serological examinations.

c.) If the virus can be transmitted horizontally in SPF mice and lesions described above reproduced, the agents will then be given to Vitamin A deficient mice and mice receiving carcinogenic compounds.

d.) If the virus can be grown, permission will be requested to send it to Microbiological Associates for identification.

INTERVAL STUDIES

a.) To continue smoke inhalation studies in the current non-pathogen free Ajax, C-57Bl, Snell, and CD-1 mice.

b.) Continue the examination of infected lung tissues from the Ajax, C-57Bl, Snell, and CD-1 smoke exposed and non-exposed groups.

c.) Look for and attempt to isolate the virus in animals to be sacrificed in February and May, 1973.

11. List publications or papers in press resulting from this or closely related work. (Append reprints or manuscripts not previously sent)..

Loosli, C.G., Buckley, R.D., Hertweck, M.S., et al. Pulmonary Response of Mice Exposed to Synthetic Smog. Annals of Occupational Hygiene; In Press.

Loosli, C.G., Buckley, R.D., Hwang-Kow, S.-Y., et al. Experimental Airborne Influenza PR8-A Infections in Germ-free Mice. IV International Symposium on Germfree Research. Academic Press, New York; In Press.

Loosli, C.G., Buckley, R.D., Hwang-Kow, S.-Y., et al. Effect of Air Pollutants on Resistance of Mice to Airborne Influenza A Virus Infection. IV International Symposium on Aerobiology, Utrecht, the Netherlands, Sept. 6, 1972; In Press.

Loosli, C.G., Stinson, S.F., Hwang-Kow, S.-Y., et al. Effect of Vitamin A Intake on the Pathology of Airborne Influenza A Virus Infection. IV International Symposium on Aerobiology, Utrecht, the Netherlands, Sept. 6, 1972; In Press.

Hung, Kuen-Shan, Hertweck, M.S., Hardy, J.D., and Loosli, C.G. Innervation of Pulmonary Alveoli of the Mouse Lung: An Electron Microscopic Study. American Journal of Anatomy 135: 447-495, 1972. Reprint sent to C.T.R.

Hung, K.-S., Hertweck, M.S., Hardy, J.D., and Loosli, C.G. Filaments in Fibroblast in Pulmonary Alveolar Wall. Proceedings, 30th Annual Meeting of the Electron Microscopy Society of America, pp. 248-249. C.J. Arceneaux, editor. Claitor's Publishing Division, Baton Rouge, 1972. Reprint sent to C.T.R.

Hung, K.-S., Hertweck, M.S., Hardy, J.D., and Loosli, C.G. Electron Microscopic Observations of Nerve Endings in the Alveolar Walls of Mouse Lungs. Abstract appeared in Amer. Rev. Resp. Dis., 1972 105, 1008. Accepted for publication by the Amer. Rev. Resp. Dis. January, 1973. Reprint sent to C.T.R.

Hung, K.-S., Hertweck, M.S., Hardy, J.D. and Loosli, C.G. Ultrastructure of Nerves and Associated Cells in Bronchiolar Epithelium of the Mouse Lung. Submitted for publication to the Journal of Ultrastructure Research. Reprint sent to C.T.R.

12. Progress report submitted separately.

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s) even if no salary requested)

Professional:	% Time	Amount
Clayton G. Loosli, M.D., Ph.D. Principal Investigator	80*	$16,350
Helgard Niewisch, D.V.M.,Res. Assoc. Path.	100	14,770
Kuen-Shan Hung, Ph.D.,Asst. Prof. Anat&Path.	30**	1,470
Virologist - to be recruited	100	11,000
Technical:		
M. Sue Hertweck, M.S., Electron Microscp.	100	11,330
Edna Stone, M.S., Histology Tech.	100	11,520
Rhoda Serebrin, B.S., Histochemist	66	6,750
Lee Strom, B.S., E.M. Tech	100	6,960
Pete Radanovich, Animal Caretaker	100	5,530
Carlos Garcia, Animal Caretaker	100	8,860
Smoking Machine Operators (3)	100	20,680
		115,220
Fringe Benefits (10%)		11,522
Sub Total for A		$126,742

B. Consumable supplies (by major categories)

Animals: 2,400 mice at $1.00	2,400
20 rabbits at $4.50	90
Food and Vivaria costs:	6,000
Electron microscopic supplies	2,500
Histopathological, Histochemical supplies	1,500
Microbiological, Virological, Immunological supplies	3,000
Reference cigarettes	650
Sub Total for B	13,240

C. Other expenses (itemize)

Maintenance of electron microscope	2,400
Service and Repairs of Equipment	2,000
Maintenance of Air Pollution Bldg. & Germfree Facility	4,000
Office and Communication costs	2,000
Publication costs	1,500
Travel, two meetings East Coast, two local meetings	1,200
Sub Total for C	$13,100
RUNNING TOTAL OF A+B+C	$156,082

D. Permanent Equipment (itemize)

CO_2 Incubator National	2,500
Sub Total for D	$2,500

E. Indirect costs (15% of A+B+C) E $23,412

TOTAL REQUEST: $181,994

* 80% effort, but only 28% salary support
** 30% effort, but only 8% salary support

14. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects:

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
USC School of Medicine Contribution	Hastings Foundation 23-5120-4092	$43,000	7-1-73 to 6-30-74
	Hughes Employees Give Once Club 23-5120-4234	$26,500	7-1-73 to 6-30-74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NONE	NONE		

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It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Name Clayton G. Loosli, M.D.

Signature [signature]

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Responsible officer of institution:

Name Dr. Z.A. Kaprielian

Title V.P. Academic Administration and Research

Signature

Telephone (213) 225-1511, x432